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14. ABSTRACT

The newly discovered RNA interference is a novel type of gene regulation mechanism, which is required for normal expression of genes. This study tests the hypothesis that breast tumor carries dysregulated RNA interference pathways, and thus, some tumor suppressor genes will be down-regulated while other genes (e.g., oncogenes) will be up-regulated, leading to tumor cell proliferation and survival. Using real time RT-PCR, we demonstrate that microRNA-21 is overexpressed in breast tumors compared to the matched normal breast tissue. Furthermore, we show that antisense oligonucleotide against microRNA-21 can suppress the endogenous microRNA-21 and causes tumor cell growth inhibition. Experiments with a xenograft carcinoma mouse model reveal that the antisense microRNA-21 oligonucleotide also inhibits tumor growth. Therefore, microRNA-21 is a potential therapeutic target for breast cancer therapy.

15. SUBJECT TERMS
RNA interference, RNAi, microRNA, breast tumorigenesis.

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Introduction

Breast cancer, like other types of cancers, is frequently caused by altered gene expressions. The newly discovered RNA interference is a novel type of gene regulation mechanism. There are two types of small RNAs that are processed by the RNA interference pathway, short interfering RNAs (siRNAs) and microRNAs (miRNAs), both of which can silence gene expression at the post-transcriptional level. While siRNAs are more or less artificial molecules, miRNAs are naturally occurring small RNA molecules. To date, over 500 human miRNAs have been identified. Accumulating evidence from different research groups including ours indicates that miRNAs play a critical role in tumor growth, cell invasion and metastasis. In this funding period, we have been focusing on miR-21 because this miR-21 is overexpressed in breast tumors compared to matched normal breast tissue.

Body

miR-21 is overexpressed in breast tumor tissues compared to the matched normal breast tissues

We used a newly released miRNA array from ABI (Forest City, CA) profiled miRNA expression in matched breast tumor specimens and found that miR-21 is overexpressed in breast tumors. As shown in Fig. 1, the miR-21 level was over 5-fold higher in tumor than in the matched normal tissues, suggesting that miR-21 is an oncogenic miRNA.

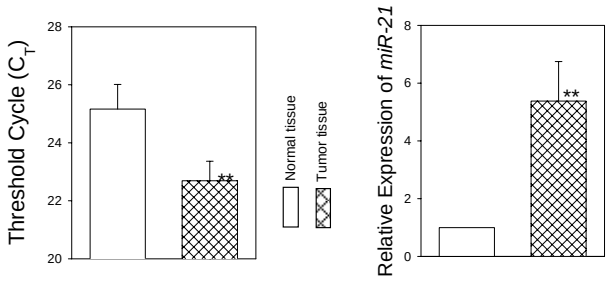

Fig.1 Expression of *miR-21* in matched normal and breast tumor tissues. Relative *miR-21* levels were expressed as CT value (**A**) or fold change after normalization to U6 RNA (**B**).

Anti-miR-21 inhibits cell growth in vitro and in vivo

To test whether miR-21 is an oncogenic miRNA, we examined the effect of suppression of miR-21 on breast tumor cell growth. Thus, we used anti-miR-21 inhibitor since this approach has been successfully used to inhibit *miR-21* (1, 2). The anti-miR-21 inhibitor is a sequence-specific and chemically modified oligonucleotide to specifically target and knockdown *miR-21* molecule. Of interest, we found that anti-miR-21 reduced cell growth in a dose dependent manner. At 50 nM, the growth inhibition by anti-miR-21 reached about 25%, at day 3 after transfection (Fig. 2). To further determine the role of miR-21 in tumor growth, we transiently transfected MCF-7 cells with anti-miR-21 or the negative control, and then injected them into mammary pads of female nude mice. We found that tumors derived from MCF-7 cells transfected with anti-miR-21 grew substantially slowly, compared to the negative control during the whole tumor growth period (3). These results strongly suggest that *miR-21* plays an important role in tumorigenesis.

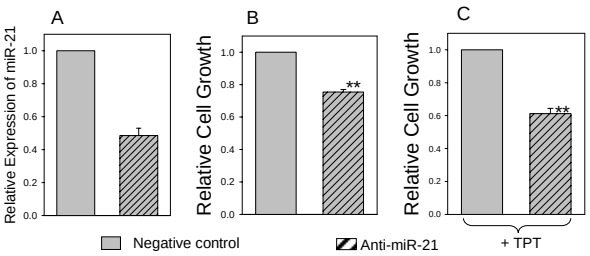

Fig.2 Inhibition of cell growth by anti-miR-21 oligonucleotides. A, Suppression of *miR-21* expression by anti-miR-21 as detected by TaqMan Real-time PCR. **B**, Cell growth inhibition. MCF-7 cells were transiently transfected with the negative control or anti-miR-21 oligonucleotides at 50 nM and then were seeded in 96 well at 2500 cell/well. Cell growth inhibition was determined by MTT assay. **C**, Cell growth inhibition in the presence of the anticancer agent topotecan (TPT) 0.1 μM . Values in both **B** and **C** are means of three separated experiments ±SE. **, p<0.01. N, negative control; A, anti-miR-21.

Anti-miR-21 increases cell apoptosis in part through targeting the tumor suppressor gene tropomyosin 1

To dissect the molecular basis underlying this *miR-21*-associated alteration of tumor growth, we searched for potential *miR-21* targets using programs available and tested several genes that are likely involved in tumorigenesis such as FasL. However, their protein levels were not affected by anti-miR-21 (not shown). Thus, we tested

whether anti-miR-21 suppresses cell growth by triggering apoptosis pathways since previous studies have suggested that *miR-21* regulates apoptosis pathways in tumor cells (2). Anti-miR-21 caused more apoptosis than the negative control in MCF-7 cells by a 4.5-fold (3). To further determine the possible involvement of apoptosis in anti-miR-21-mediated growth inhibition we treated transfected cells with the general caspase inhibitor Z-VAD-fmk, which was able to reverse the growth inhibition caused by anti-miR-21, suggesting that increased apoptosis in the anti-miR-21-treated MCF-7 cells is at least in part responsible for the observed growth inhibition (3). Using the proteomic approach (Fig.3), we successfully identified the tumor suppressor gene tropomyosin 1 (TPM1) as a direct miR-21 target (4). Therefore, this may explain in part why miR-21 plays a role in breast cancer.

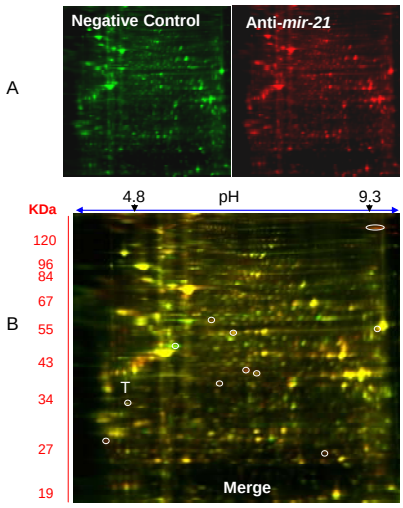

Fig.3 Identification of TMP1 as a miR-21 target by 2-DIGE. Protein was labeled with Cy3 (Green) for negative control and Cy5 (Red) for anti-miR-*21*, respectively. **A**, Protein profiles of tumor samples treated with negative control or anti-miR-*21*. **B**, An gel picture after merge. Protein spots in red are presumably due to upregulation by anti-miR-*21* and 10 spots are shown by a circle. T, TPM1

Effect of Dicer on tumor cell growth

Dicer1 is a key enzyme regulating siRNA/miRNA biogenesis pathway. Thus we overexpressed Dicer in MCF-7 cells. Our surprise, Dicer overexpression in fact caused growth inhibition. This is probably due to that fact that Dicer affects so many miRNAs. Overexpression of Dicer may lead to increases in some of pro-apoptosis inducing miRNAs.

Key Research Accomplishments

- MicroRNA profiling indicates that *miR-21* was highly overexpressed in breast tumors compared to the matched normal breast tissues
- Anti-miR-21 suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model.
- This anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation.
- miR-21 directly targets the tumor suppressor gene TPM1.
- Dicer seems to have deleterious effect on cell growth when it is overexpressed.
- Together, these results suggest that *miR-21* functions as an oncogene and modulates tumorigenesis and thus, *miR-21* may serve as a novel therapeutic target.

Reportable Outcomes

Abstracts

Abstract presented in 2006 AACR meeting entitled "Suppression of tumor growth by anti-miRNA21" April, 2006, Washington DC.

Abstract presented in 2007 AACR meeting entitled "miRNA21 targets the tumor suppressor gene tropomyosin" April, 2007, Los Angeles, CA.

Publications

1: Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem. 2007 May 11;282(19):14328-36. Epub 2007 Mar 15. PMID: 17363372 [PubMed - in process]

2: Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. Oncogene. 2007 Apr 26;26(19):2799-803. Epub 2006 Oct 30. PMID: 17072344 [PubMed - indexed for MEDLINE]

Conclusions

We have demonstrated that miR-21 is overexpressed in breast tumor compared to the matched normal breast tissues. More importantly, suppression of miR-21 by antisense

oligonucleotide inhibits breast tumor growth. Therefore, the antisense miR-21 oligonucleotide may prove a potent therapeutic agent. Future work will be to understand molecular mechanism by which miR-21 impacts on breast cancer.

A list of personnel supported by the award

Katie DeClerk (researcher)
Shoumin Zhu (Postdoc)

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2. Chan, J. A., Krichevsky, A. M., and Kosik, K. S. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res, *65:* 6029-6033, 2005.
3. Si, M. L., Zhu, S., Wu, H., Lu, Z., Wu, F., and Mo, Y. Y. miR-21-mediated tumor growth. Oncogene, *26:* 2799-2803, 2007.
4. Zhu, S., Si, M. L., Wu, H., and Mo, Y. Y. MicroRNA-21 Targets the Tumor Suppressor Gene Tropomyosin 1 (TPM1). J Biol Chem, *282:* 14328-14336, 2007.

Appendix (Supporting data)

2 abstracts
2 publications

Suppression of tumor growth by anti-miRNA-21

Min-Liang Si, Hailong Wu, Zhaohui Lu, Fangting Wu and Yin-Yuan Mo
Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL

Since the discovery of the first microRNA (miRNA), lin-4, in the nematode Caenorhabditis elegans, many more of these short regulatory RNAs have been identified in flowering plants, worms, flies, fish, frogs and mammals. It is predicted that about 2% of the known human genes encode miRNAs and over 300 miRNAs have been identified to date. It appears that miRNAs could have a fundamental effect on cellular pathways. It has been recently shown that miRNAs are aberrantly expressed in cancer, suggesting their roles in oncogenesis. Using miRNA microarray and TaqMan real-time miRNA array analysis, we found that miRNAs such as miRNA-21 were highly expressed in both breast tumor cell line MCF-7, and tumor tissues from breast cancer patients compared to normal tissues. In contrast, expression of miRNA205, miR125b-1, miRNA145 was lower in tumor tissues than in normal tissues. We also found that MCF-7 cells transfected with an anti-sense oligonucleotide against miRNA-21 grew significantly slowly than the control. Consistent with the in vitro growth data, experiments with a xenograft mouse model revealed that tumors derived from MCF-7 cells transfected with the anti-sense miRNA-21 were also smaller than those derived from the control. Further studies indicated that the anti-sense miRNA-21 affected cell growth through caspase-mediated apoptosis pathways. Together, these results suggest that dysregulation of miRNAs may play an important role in tumorigenesis. (Supported in part by BC045418 from DOD and by CA102630 from NIH)

2007 AACR meeting

Mir-21 directly targets the tumor suppressor gene tropomyosin 1 (TPM1)

Shoumin Zhu, Min-Liang Si and Yin-Yuan Mo
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MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNA molecules, which regulate expression of target genes through translational repression or mRNA cleavage. Although aberrant expression of miRNAs in various human cancers suggests a role for miRNAs in tumorigenesis, it remains largely unclear as to how a specific miRNA affects tumor growth because our understanding of miRNA target genes is limited. In this study, we profiled miRNA expression in matched normal breast and breast tumor tissues by TaqMan real-time PCR miRNA array methods. Consistent with previous findings, we found that *mir-21* was highly overexpressed in breast tumors compared to the matched normal breast tissues among 157 human miRNAs analyzed. To better evaluate the role of *mir-21* in tumorigenesis, we transfected breast cancer MCF-7 cells with antisense *mir-21* oligonucleotide and found that anti-*mir-21* suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model. To determine *mir-21* target genes, we performed two dimensional differentiation in gel electrophoresis (2-DIGE) and identified tropomyosin 1 (TPM1) as a potential *mir-21* target. In agreement with this, by searching miRNA target databases, we found that there was a putative *mir-21* binding site at the 3'-untranslational region (3'-UTR) of TPM1 variants 1 and 5. To further confirm TPM1 as a *mir-21* target, we cloned the 3'-UTR of TPM1 variant 1 into a luciferase reporter and found that while *mir-21* downregulated, anti-*mir-21* upregulated the luciferase activity of TPM1. In contrast, *mir-21* had no effect on TPM1-V4 which lacks the *mir-21* binding site. Furthermore, deletion of the *mir-21* binding site abolished the effect of *mir-21* on the lucfierase activity, suggesting that this *mir-21* binding site is critical. Western blot with the cloned TPM1-V1 carrying the 3'-UTR indicated that TPM1-V1 protein level was also regulated by *mir-21*, whereas real-time RT-qPCR revealed no difference at the mRNA level, suggesting that *mir-21*-mediated regulation of TPM1 is at the translational level. As a tumor suppressor, TPM1 has been shown to affect tumor growth and metastasis. Thus, downregulation of TMP1 by *mir-21* may explain at least in part why suppression of *mir-21* can inhibit tumor growth, supporting the notion that *mir-21* function as an oncogene. Therefore, *mir-21* is a potential novel therapeutic target for cancer therapy. (supported by grants BC045418 and BC052294 from DOD).

Oncogene (2006), 1–5

www.nature.com/onc

SHORT COMMUNICATION

miR-21-mediated tumor growth

M-L Si, S Zhu, H Wu, Z Lu, F Wu and Y-Y Mo

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MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNA molecules that regulate gene expression post-transcriptionally. Although aberrant expression of miRNAs in various human cancers suggests a role for miRNAs in tumorigenesis, it remains largely unclear as to whether knockdown of a specific miRNA affects tumor growth. In this study, we profiled miRNA expression in matched normal breast tissue and breast tumor tissues by TaqMan real-time polymerase chain reaction miRNA array methods. Consistent with previous findings, we found that *miR-21* was highly overexpressed in breast tumors compared to the matched normal breast tissues among 157 human miRNAs analysed. To better evaluate the role of *miR-21* in tumorigenesis, we transfected breast cancer MCF-7 cells with anti-miR-21 oligonucleotides and found that anti-miR-21 suppressed both cell growth *in vitro* and tumor growth in the xenograft mouse model. Furthermore, this anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation, which could be in part owing to downregulation of the antiapoptotic Bcl-2 in anti-miR-21-treated tumor cells. Together, these results suggest that *miR-21* functions as an oncogene and modulates tumorigenesis through regulation of genes such as *bcl-2* and thus, it may serve as a novel therapeutic target.
Oncogene advance online publication, 30 October 2006; doi:10.1038/sj.onc.1210083

Keywords: miRNA; *miR-21*; post-transcriptional regulation; Bcl-2; MCF-7

Introduction

MicroRNAs (miRNAs) are a class of naturally occurring small non-coding RNAs that control gene expression by targeting mRNAs for translational repression or cleavage (Pillai, 2005; Zamore and Haley, 2005). It is predicted that miRNAs comprise 1–5% of animal genes (Berezikov *et al*., 2005). miRNAs are transcribed as long primary transcripts in the nucleus and are subsequently cleaved to produce stem loop structured precursor molecules of ~70 nt in length (pre-miRNAs) by Drosha (Kim, 2005), which are then exported to the cytoplasm, where the RNase III enzyme Dicer further processes them into mature miRNAs (~22 nucleotides). Thus, miRNAs are related to short interfering RNAs, but they have distinct pathways (Bartel, 2004; Fitzgerald, 2005). Since the discovery of *lin-4* in *Caenorhabditis elegans* (Lee *et al*., 1993; Wightman *et al*., 1993), thousands of miRNAs have been identified to date in a variety of organisms (http://microrna.sanger.ac.uk/).

As a new layer of gene regulation mechanism, miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation and apoptosis (Chen *et al*., 2004; Croce and Calin, 2005). Thus, deregulation of miRNAs would alter the normal cell growth and development, leading to a variety of disorders including human cancer. For instance, about 65% of investigated patients suffering from B-cell chronic lymphocytic leukemia (CLL) have been reported to show a deletion located at chromosome 13q14 where the *miR-15* and *miR-16* genes are located and are under-represented in many B-CLL patients (Calin *et al*., 2002). Of interest, miRNA-containing regions are often located at fragile sites or in repetitive genomic sequences (Calin *et al*., 2004). Deregulation of other miRNAs has also been reported in different cancers (Michael *et al*., 2003; Metzler *et al*., 2004; Eis *et al*., 2005), indicating that there is a direct correlation between aberrant expression of miRNAs and human malignancy. However, although miRNAs have been the object of extensive research in recent years, the molecular basis of miRNA-mediated gene regulation is not fully understood and their role in tumorigenesis remains largely to be determined yet.

In this study, we found that *miR-21* was overexpressed in breast tumor specimens, consistent with the previous report (Iorio *et al*., 2005). Importantly, anti-miR-21 oligonucleotides suppress both cell growth *in vitro* and tumor growth *in vivo*, which is associated with increased apoptosis and downregulation of the antiapoptotic protein Bcl-2.

Results and discussion

miR-21 *is overexpressed in breast tumor tissues compared to matched normal breast tissues*

Previous studies have shown that several miRNAs are aberrantly expressed in various types of cancers by

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miRNA array or Northern blot (Calin *et al*., 2002, 2004; Michael *et al*., 2003; Metzler *et al*., 2004; Eis *et al*., 2005). In this study, we profiled miRNA expression in matched normal breast and breast tumor tissues by TaqMan real-time polymerase chain reaction (PCR) using a newly released miRNA array from ABI (Forest City, CA, USA). The array carries specific primer sets that allow for detection of 157 mature human miRNAs. This method uses stem–loop reverse transcription (RT) primers; it is specific for detection of mature miRNAs (Chen *et al*., 2005; Lao *et al*., 2006). Furthermore, this method is very sensitive and is able to analyse miRNA expression in a single cell (Tang *et al*., 2006). We used U6 RNA for normalization of expression in different samples. From a total of five pairs of matched advanced breast tumor tissue specimens, *miR-21* was the most abundantly expressed miRNA among all miRNAs in this array and moreover, the level of *miR-21* was much higher in the tumor tissues than in the matched normal tissues (Figure 1a). As one C_T (threshold cycle) unit is equivalent to $\sim$2-fold difference (Chen *et al*., 2005), this conversion would result in over a five-fold increases in *miR-21* levels for tumor tissues compared to the matched normal tissues after normalization to U6 RNA (Figure 1b), consistent with the previous report (Iorio *et al*., 2005). Furthermore, using the individual *miR-21* primer set, we were able to confirm these results in more matched breast tumor samples (not shown).

Anti-miR-21 inhibits cell growth in vitro

To test whether *miR-21* may function as an oncogene, we examined the effect of suppression of *miR-21* on breast tumor cell growth. Thus, we used anti-miR-21 inhibitor as this approach has been successfully used to inhibit *miR-21* (Chan *et al*., 2005; Cheng *et al*., 2005). The anti-miR-21 inhibitor is a sequence-specific and chemically modified oligonucleotide to specifically target and knockdown *miR-21* molecule. TaqMan real-time PCR revealed that anti-miR-21 significantly reduced *miR-21* level (Figure 2a), suggesting that anti-miR-21 is efficiently introduced into the cells and knock down *miR-21*. This is probably due to the formation of highly stable complexes of *miR-21* with anti-miR-21 that prevents miRNA detection by TaqMan real-time PCR. Of interest, we found that anti-miR-21 reduced cell growth in a dose-dependent manner. At 50 nM, the growth inhibition by anti-miR-21 reached about 25%, at day 3 after transfection (Figure 2b); this result was also in agreement with the previous report that *miR-21* inhibitors decrease human glioblastoma cell survival (Chan *et al*., 2005). To further assess the effect of anti-miR-21 on cell growth, we treated the transfected cells with the anticancer drug topotecan (TPT) that is known to inhibit DNA topoisomerase I and cause DNA damage (Tanizawa *et al*., 1994). Anti-miR-21-mediated cell growth inhibition was increased up to 40% when the transfected cells were treated with 0.1 μM TPT (Figure 2c). Therefore, anti-miR-21 can inhibit cell growth *in vitro*. These results also suggest that suppression of *miR-21* can sensitize tumor cells to anticancer agents.

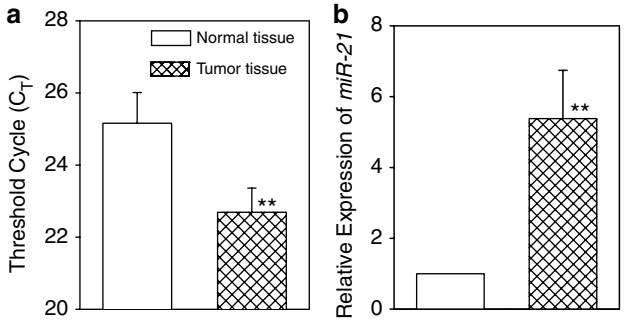

Figure 1 Expression of *miR-21* in matched normal and breast tumor tissues. Relative *miR-21* levels were determined by TaqMan miRNA assays (see Supplementary materials for detail), expressed as C_T (**a**) or fold change after normalization to U6 RNA (**b**). For RT reactions, 10 ng total RNA was used in each reaction (15 μl) and mixed with corresponding TaqMan miRNA assays RT primer (3 μl). The RT reaction was performed at the following conditions: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, and then hold on 4°C. After the RT reaction, the cDNA products were diluted at 15, 150 and 1500 ×, respectively, and 1.33 μl diluted cDNA was used for PCR reaction along with TaqMan primer (2 μl). The PCR reaction was carried out at 95°C for10 min, followed by 40 cycle of 95°C for 15 s and 60°C for 60 s. Values are means of five pairs of matched breast tumor samples $\pm$s.e. $^{**}P<0.01$.

Anti-miR-21 inhibits tumor growth in the xenograft carcinoma mouse model

Although it has previously been shown that there is a direct correlation between aberrant expression of *miR-21* and breast cancer (Iorio *et al*., 2005), it is not clear whether suppression of *miR-21* alone will affect tumorigenesis. Therefore, we transiently transfected MCF-7 cells with anti-miR-21 or the negative control, and then injected them into mammary pads of female nude mice. Of considerable interest, we found that tumors derived from MCF-7 cells transfected with anti-miR-21 grew substantially slowly, compared to the negative control during the whole tumor growth period (Figure 3a). By day 28 when tumors were harvested, average weight for tumors derived from cells transfected with anti-miR-21 was only about half of those derived from the cells transfected with the negative control (Figure 3b). Immunostaining with the anti-Ki-67 indicated that the reduced tumor growth is likely due to a lower proliferation caused by anti-miR-21 because Ki-67 staining was much weaker for anti-miR-21 than for the negative control (Figure 3c). These results strongly suggest that *miR-21* plays an important role in tumorigenesis. To test how long suppression of *miR-21* by anti-miR-21 in tumors can sustain, we measured the miR-21 levels. We found that the suppression effect lasted up to 2 weeks (Supplementary materials), suggesting that the initial suppression of *miR-21* is sufficient to inhibit tumor growth.

Of interest, the inhibitory effect of anti-miR-21 on tumor growth (Figure 3b) is greater ($\sim$50%), compared to its inhibitory effect on cell growth *in vitro* ($\sim$25%) (Figure 2b). Although the observation time for tumor growth is longer than *in vitro* cell growth inhibition assays, which could explain in part the difference, other

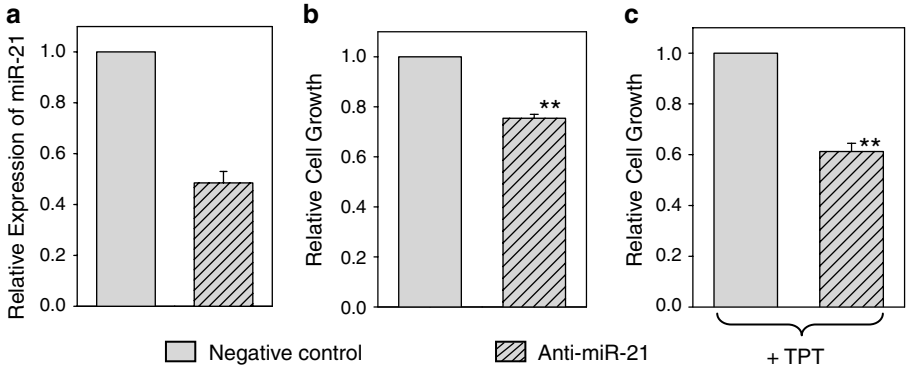

Figure 2 Inhibition of cell growth by anti-miR-21 oligonucleotide. (**a**) Suppression of *miR-21* expression by anti-miR-21 as detected by TaqMan real-time PCR. (**b**) Cell growth inhibition. MCF-7 cells were transiently transfected with the negative control or anti-miR-21 oligonucleotide at 50 nM and then were seeded in 96 well at 2500 cells/well. The cells were allowed to grow for 3 days before MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) assay, as described previously (Mo *et al*., 2004). (**c**) Cell growth inhibition in the presence of the anticancer agent TPT. Cells were first transfected with 50 nM of negative control or anti-miR-21 and then treated with 0.1 μM of TPT for 3 days. Values in both (**b**) and (**c**) are means of three separated experiments $\pm$s.e. $^{**}P<0.01$.

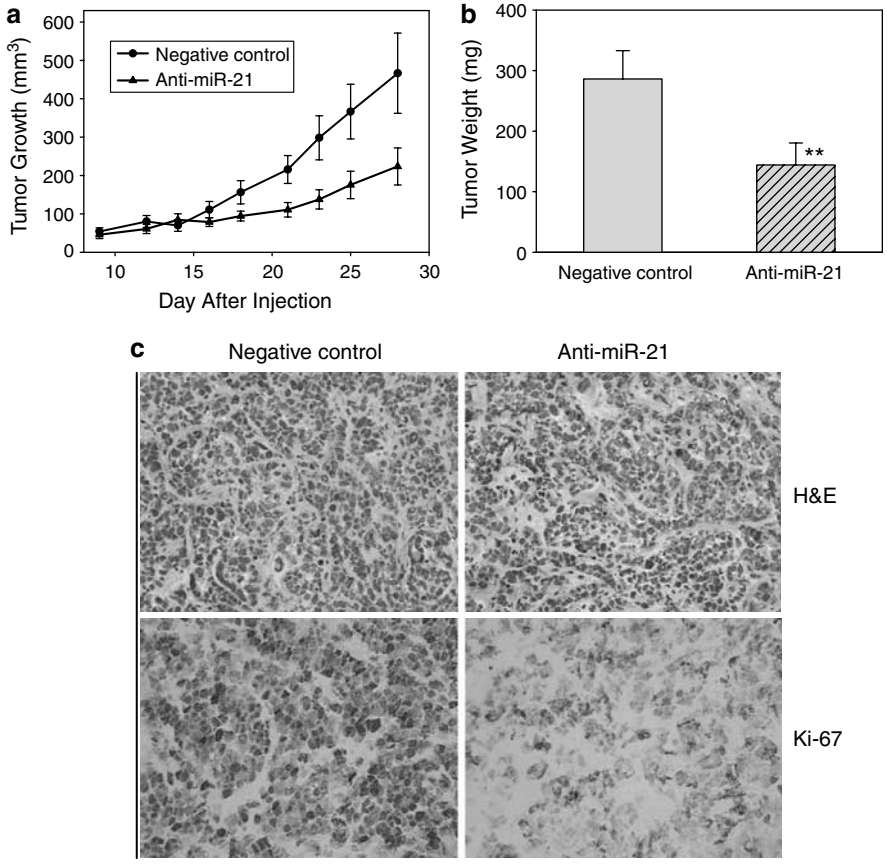

Figure 3 Suppression of tumor growth by anti-*miR-21* oligonucleotide. (**a**) Tumor growth curves measured after injection of MCF-7 cells transfected with either the negative control or anti-miR-21 oligonucleotides. The tumor volume was calculated using the formula volume $= D \times d^2 \times \pi/6$ (Zhang *et al*., 2002), where D is the longer diameter, d is the shorter diameter. (**b**) Tumor weight. Values in (**a**) and (**b**) are means of tumor volume or weight $\pm$s.e. (negative control, $n=14$; anti-miR-21, $n=16$). $^{**}P<0.01$. (**c**) Tumors derived from anti-miR-21-transfected cells revealed a lower level of Ki-67 antigen than the negative control.

factors could also contribute to this difference. For instance, stress from the tumor microenvironment, such as hypoxia, may enhance the inhibitory effect of the anti-miR-21. This appears to be in agreement with the finding that other stresses, such as DNA damage caused by TPT, can increase the inhibitory effect mediated by

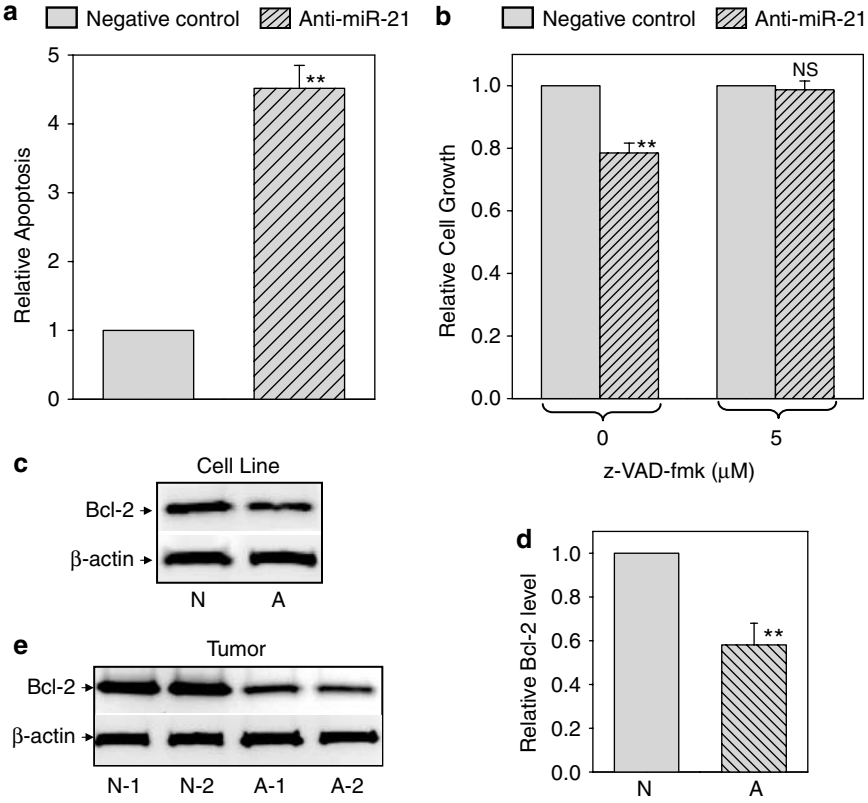

Figure 4 Anti-*miR-21*-induced apoptosis and downregulation of Bcl-2. (**a**) Detection of apoptosis in MCF-7 cells transfected with anti-miR-21 compared to the negative control using cell death detection ELISA^{plus} kit (Hoffmann-La Roche Ltd, Basel, Switzerland). (**b**) Suppression of anti-miR-21-induced growth inhibition by the general caspase inhibitor Z-VAD-fmk. MCF-7 cells were transfected with the negative control or anti-miR-21 as in Figure 2a and then the caspase inhibitor was added to the transfected cells 1 day after transfection. After 3 days later, cell growth inhibition was determined. (**c**–**e**) Expression of Bcl-2 protein in anti-miR-21 in MCF-7 cells (**c** and **d**) and tumors derived from MCF-7 cells transfected with the negative control or anti-miR-21 (**e**) as detected by Western blot. N-1 and N-2 are tumors 1 and 2 derived from the negative control-treated MCF-7 cells, respectively; A-1 and A-2 are tumors 1 and 2 derived from the anti-miR-21-treated MCF-7 cells, respectively. Values in (**a**), (**b**) and (**d**) are means of three separate experiments $\pm$s.e. $^{**}P<0.01$. NS, not significant; N, negative control; A, anti-miR-21.

anti-miR-21 (Figure 2c). Alternatively, anti-miR-21 could also affect genes that are linked to other tumorigenesis factors, which might explain in part why more inhibition for anti-miR-21 was seen in tumors than cell growth *in vitro*.

Anti-miR-21 increases cell apoptosis which is associated with downregulation of bcl-2 *expression*

To dissect the molecular basis underlying this *miR-21*-associated alteration of tumor growth, we searched for potential *miR-21* targets using programs available (e.g., http://microrna.sanger.ac.uk/targets/v2/; http://genes.mit.edu/cgi-bin/targetscan_lookup2.pl?KEYWORD=miR-21) and tested several genes that are likely involved in tumorigenesis, such as FasL. However, they were not affected by anti-miR-21 (Supplementary materials). Thus, we tested whether anti-miR-21 suppresses cell growth by triggering apoptosis pathways as previous studies have suggested that *miR-21* regulates apoptosis pathways in tumor cells (Chan *et al*., 2005). Consistent with the previous report for glioblastoma cells (Chan *et al*., 2005), but contrary to the results in HeLa cells (Cheng *et al*., 2005), we found that anti-miR-21 caused more apoptosis than the negative control in MCF-7 cells by a 4.5-fold (Figure 4a). To further determine the possible involvement of apoptosis in anti-miR-21-mediated growth inhibition, we treated transfected cells with the general caspase inhibitor Z-VAD-fmk. As shown in Figure 4b, Z-VAD-fmk was able to reverse the growth inhibition caused by anti-miR-21, suggesting that increased apoptosis in the anti-miR-21-treated MCF-7 cells is at least in part responsible for the observed growth inhibition. Furthermore, we detected a lower level of Bcl-2 protein in the anti-miR-21-transfected MCF-7 cells (Figure 4c and d) as well as tumors derived from the MCF-7 cells transfected with anti-miR-21 (Figure 4e). Given that suppression level of Bcl-2 *in vivo* (Figure 4e) is greater than that *in vitro* (Figure 4c), it is possible that tumor microenvironment may enhance downregulation of Bcl-2 in the anti-miR-21-treated tumors. We also tested other apoptosis-related proteins such as p53 and PUMA, and found no difference between the negative control and anti-miR-21 (Supplementary materials). Thus, the induction of apoptosis by

anti-miR-21 is possibly in part owing to downregulation of Bcl-2. We also examined *bcl-2* mRNA by RT–PCR and found that *bcl-2* mRNA was decreased in the anti-miR-21-treated cells (Supplementary materials), suggesting that *miR-21* may regulate *bcl-2* expression indirectly. Although we cannot exclude the possibility that anti-miR-21 may cause degradation of *bcl-2* mRNA, one possibility would be that anti-miR-21 suppresses expression of a gene(s) that negatively regulates *bcl-2* expression. Therefore, identification of direct *miR-21* targets may provide new insight into how *miR-21* controls expression of genes involved in apoptosis pathways including *bcl-2*.

In summary, we show that *miR-21* is overexpressed in breast tumor tissues and anti-miR-21 inhibits both cell growth *in vitro* and tumor growth *in vivo*. This is possibly owing to increased apoptosis associated with downregulation of *bcl-2* expression. As experiments with the xenograft carcinoma model indicate that one transient transfection with anti-miR-21 is sufficient to cause substantial inhibition of tumor growth, this raises the possibility that anti-miR-21 may have potential therapeutic value. Indeed, anti-miRNA oligonucleotides can stay a relatively long period of time in animals (Krutzfeldt *et al*., 2005). Therefore, miRNAs, in particular *miR-21*, may serve as potential targets for cancer therapy.

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MicroRNA-21 Targets the Tumor Suppressor Gene Tropomyosin 1 (*TPM1*)*

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MicroRNAs are small noncoding RNA molecules that control expression of target genes. Our previous studies show that *mir-21* is overexpressed in tumor tissues compared with the matched normal tissues. Moreover, suppression of *mir-21* by antisense oligonucleotides inhibits tumor cell growth both *in vitro* and *in vivo*. However, it remains largely unclear as to how *mir-21* affects tumor growth, because our understanding of *mir-21* targets is limited. In this study, we performed two-dimensional differentiation in-gel electrophoresis of tumors treated with anti-*mir-21* and identified the tumor suppressor tropomyosin 1 (TPM1) as a potential *mir-21* target. In agreement with this, there is a putative *mir-21* binding site at the 3′-untranslated region (3′-UTR) of TPM1 variants V1 and V5. Thus, we cloned the 3′-UTR of TPM1 into a luciferase reporter and found that although *mir-21* down-regulated the luciferase activity, anti-*mir-21* up-regulated it. Moreover, deletion of the *mir-21* binding site abolished the effect of *mir-21* on the luciferase activity, suggesting that this *mir-21* binding site is critical. Western blot with the cloned TPM1-V1 plus the 3′-UTR indicated that TPM1 protein level was also regulated by *mir-21*, whereas real-time quantitative reverse transcription-PCR revealed no difference at the mRNA level, suggesting translational regulation. Finally, overexpression of TPM1 in breast cancer MCF-7 cells suppressed anchorage-independent growth. Thus, down-regulation of TPM1 by *mir-21* may explain, at least in part, why suppression of *mir-21* can inhibit tumor growth, further supporting the notion that *mir-21* functions as an oncogene.

MicroRNAs (miRNAs)[2] are a class of naturally occurring small noncoding RNAs that regulate gene expression by targeting mRNAs for translational repression or cleavage (1, 2). Like protein-coding mRNAs, miRNAs are transcribed as long primary transcripts in the nucleus. However, unlike protein-coding mRNAs, miRNAs are subsequently cleaved to produce stem-loop-structured precursor molecules of ~70 nucleotides in length (pre-miRNAs) by the nuclear RNase III enzyme Drosha (3). The pre-miRNAs are then exported to the cytoplasm, where the RNase III enzyme Dicer further processes them into mature miRNAs (~22 nucleotides). Thus, miRNAs are related to, but distinct from, short inferring RNAs (siRNAs) (4, 5). A key difference between siRNAs and miRNAs is that siRNAs require almost identical sequences to targets to exert their silencing function, whereas miRNAs bind through partial sequence homology to the 3′-untranslated region (3′-UTR) of target genes. Because of this unique feature, a single miRNA has multiple targets. Thus, miRNAs could regulate a large fraction of protein-coding genes, and as high as 30% of all genes could be miRNA targets (6).

As a new layer of gene regulation mechanism, miRNAs have diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis (7, 8). Hence, deregulation of miRNA expression may lead to a variety of disorders. Aberrant expression of miRNAs in cancer has been well documented (7). Apparently, miRNAs may function as tumor suppressors or oncogenes by targeting oncogenes or tumor suppressor genes (9). In this regard, tumor-suppressive miRNAs are usually underexpressed in tumors. For instance, *let-7* is down-regulated in lung cancer (10, 11). Furthermore, more than 60% of investigated patients suffering from B-cell chronic lymphocytic leukemia (B-CLL) have been reported to show a deletion at chromosome 13q14 where the *mir-15* and *mir-16* genes are located; these genes are under-represented in many B-CLL patients (12). Deregulation of miRNAs has also been reported in many other types of cancers. However, although miRNAs have been the subject of extensive research in recent years, the molecular basis of miRNA-mediated gene regulation and the effect of these genes on tumor growth remain largely unknown because of our limited understanding of miRNA target genes.

Identification of miRNA target genes has been a great challenge. Computational algorithms have been the major driving force in predicting miRNA targets (13–15). The approaches are mainly based on base pairing of miRNA and target gene 3′-UTR, emphasizing the location of miRNA complementary elements in 3′-UTR of target mRNAs, the concentration in the seed (6–8 bp) of continuous Watson-Crick base pairing in the 5′ proximal half of the miRNA, and the phylogenetic conservation of the complementary sequences in 3′-UTRs of ortholo-

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[2] The abbreviations used are: miRNA, microRNA; siRNA, short interfering RNA; RT, reverse transcription; qRT, quantitative reverse transcription; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; TPM, tropomyosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; 5-aza-dC, 5-aza-2′-deoxycytidine; UTR, untranslated region; 2-DIGE, two-dimensional differentiation in-gel.

gous genes. However, evidence suggests that perfect seed pairing may not necessarily be a reliable predictor for miRNA-target interactions (16), which may explain why many predicted target sites are nonfunctional. A recent study also suggests that there may be at least three types of miRNA-mRNA interactions in mammals (17). Hence, with few exceptions, large portion of the physiologic targets for miRNAs remain to be identified or verified experimentally.

In this study, we analyzed tumors derived from breast cancer MCF-7 cells treated with antisense *mir-21* oligonucleotide (anti-*mir-21*) or the negative control by two-dimensional differentiation in-gel (2-DIGE) and identified the tumor suppressor tropomyosin 1 (TPM1) as a putative *mir-21* target. Subsequent experiments confirmed that *mir-21* down-regulated expression of TPM1, whereas anti-*mir-21* up-regulated its expression through the *mir-21* binding site at the 3′-UTR region. Furthermore, ectopic expression of TPM1 suppressed anchorage-independent growth.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 cells (obtained from American Type Cell Collection, Manassas, VA) were grown in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml (Cambrex). MCF10A cells (ATCC) were grown in serum-free mammary epithelial growth medium (from Cambrex) supplemented with 100 ng/ml cholera toxin (EMD Biosciences, San Diego, CA). 293T cells (ATCC) were grown in Dulbecco's modified Eagle's medium (Cambrex) supplemented with 10% fetal bovine serum. All cells were incubated at 37 °C in a humidified chamber supplemented with 5% CO_2.

Reagents—Anti-*mir-21* (AM17000, ID No. AM10206) and the negative control (AM17010) were purchased from Ambion (Austin, TX). Anti-TPM1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Transfection—Transfection of MCF-7 cells was performed with Optifect reagent (Invitrogen) following the manufacturer's protocol. Briefly, the cells were seeded in 6-well plates at 30% confluence on the day before transfection. Three μg of TPM1-expressing plasmid or control vector was used for each transfection in antibiotic free Opti-MEM medium (Invitrogen). Transfection of 293T cells was performed using the calcium phosphate method as described previously (18). The negative control oligonucleotide or anti-*mir-21* oligonucleotide (both from Ambion) at 50 nM or 3 μg of appropriate plasmid (otherwise indicated) was used for each transfection. Transfection efficiency was monitored by spiking GFP-expressing vector or β-galactosidase-expressing vector when necessary.

Detection of Mature mir-21 by TaqMan Real-time PCR—TaqMan miRNA assays (ABI, Forest City, CA) used the stem-loop method (19, 20) to detect the expression level of mature *mir-21*. For RT reactions, 10 ng total RNA was used in each reaction (15 μl) and mixed with the RT primer (3 μl). The RT reaction was carried out under the following conditions: 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min; and then held on 4 °C. After the RT reaction, the cDNA products were diluted at 150×, and 1.33 μl of the diluted cDNA was used for PCR reac-

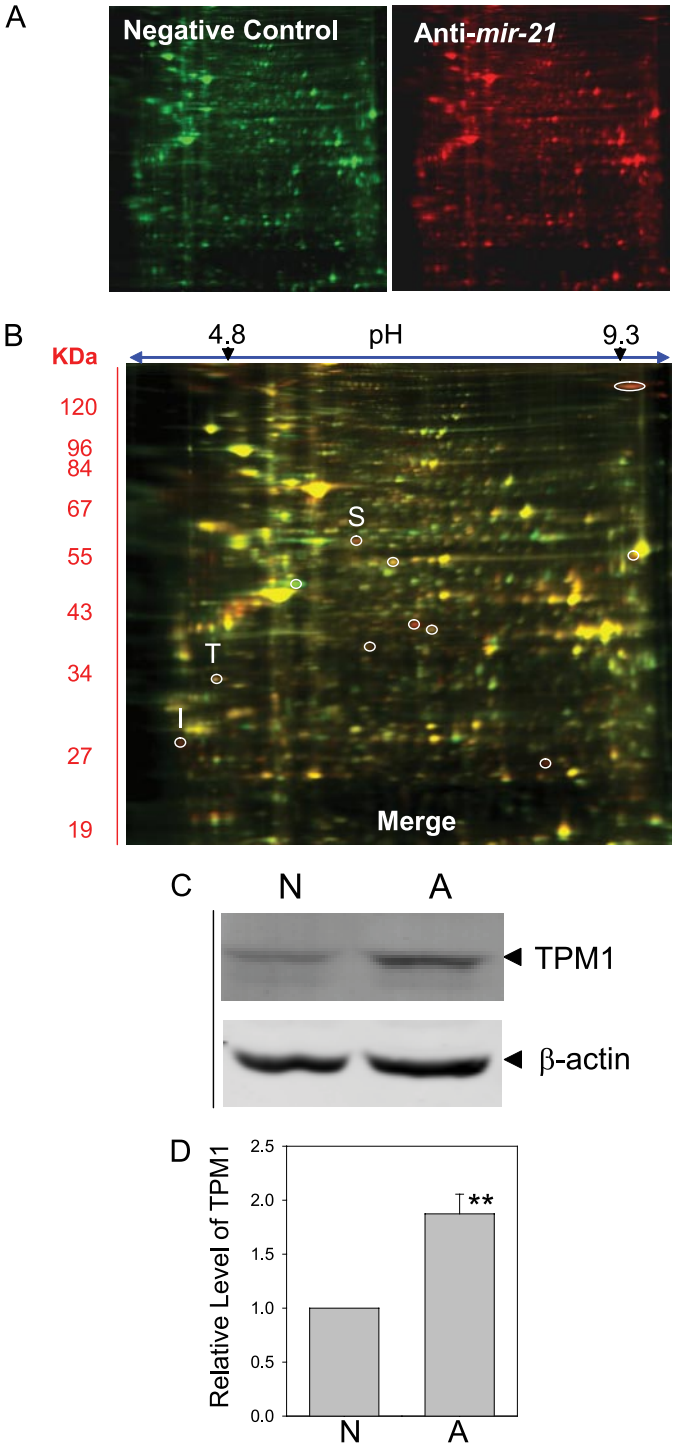

FIGURE 1. **Identification of differentially expressed proteins from tumors treated with anti-*mir-21* or negative control by 2-DIGE.** Tumors were harvested, frozen and processed as described under "Experimental Procedures." Protein was labeled with Cy3 (*green*) for negative control and Cy5 (*red*) for anti-*mir-21*, respectively. Isoelectric focusing was carried out at pH 3–10, and the two-dimensional separation was carried out in an 8–14% gradient SDS-PAGE. *A*, protein profiles of tumor samples treated with negative control or anti-*mir-21*. *B*, gel image revealing differential expression of proteins in the control and treated samples after merging. Protein spots shown in *red* are presumably due to up-regulation by anti-*mir-21*, and 10 such spots are *circled*. The molecular weight and pH markers are also indicated. *T*, TPM1; *S*, SELENBP1; *I*, ITGB4BP. *C* and *D*, up-regulation of the endogenous TPM1 in tumor samples by anti-*mir-21* as detected by Western blot. Values in *D* are means of three separate experiments ± S.E. **, $p < 0.01$. *N*, negative control; *A*, anti-*mir-21*.

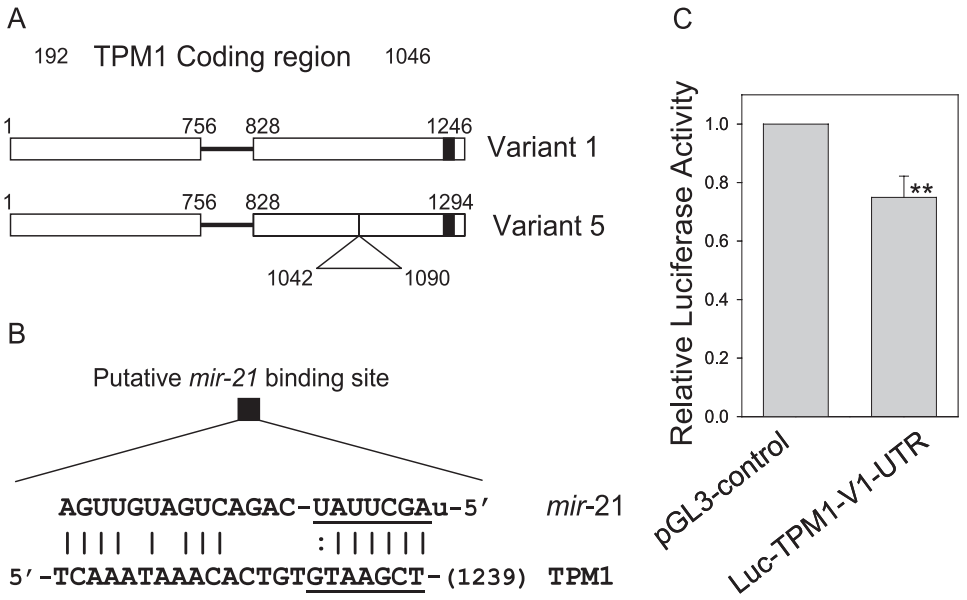

FIGURE 2. **Down-regulation of luciferase activity of Luc-TPM1-V1-UTR.** *A*, schematic description of TPM1 variants 1 (GenBank™ accession number NM_001018005.1) and 5 (GenBank™ accession number NM_000366.5). The coding region is shown in the *open box* from nucleotides 192–1406. *B*, alignment of the TPM1 UTR *mir-21* binding site from variants 1 and 5 with *mir-21*, displayed in 3′ to 5′. *C*, luciferase activity of Luc-TPM1-V1-UTR compared with that of the pGL3 control vector, which was carried out in 293T cells. **, $p < 0.01$.

tion along with TaqMan primers (2 μl). The PCR reaction was conducted at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s in the ABI 7500 real-time PCR system. The real-time PCR results were analyzed and expressed as relative miRNA expression of C_T (threshold cycle) value, which was then converted to fold changes (19). The RT primer, PCR primers, and TaqMan probe for *mir-21* (19) were purchased from ABI. U6 or 5S RNA was used for normalization.

Detection of TPM1 mRNA—To detect relative levels of TPM1 transcription, qRT-PCR was performed using the Cyber Green method under the following conditions: 94 °C for 3 min followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 0.5 min. PCR primers were TPM1-5.1, sense, 5′-CTCTCAACGATATGACTTCCA-3′, and TPM1-3.1, antisense, 5′-TTTTTTTAGCTTACACAGTGTT-3′. Both were purchased from Sigma-Genosys (Woodland, TX).

Constructs—To construct a plasmid expressing *mir-21*, we first modified pCMV-Myc (Clontech, Mountain View, CA) by deleting the Myc tag by PCR. We then amplified a 500-bp DNA fragment carrying pre-*mir-21* from MCF10A genomic DNA using PCR primers *mir-21*-5.1, 5′-GAATTCTGATTGAACTTGTTCATTTT-3′ where the EcoRI site is underlined, and *mir-21*-3.1, 5′-GGTACCAATTAAGACTATCCCCATTTCTCCA-3′, where the KpnI site is underlined. The amplified fragment was first cloned into pCR8 (Invitrogen) and was subsequently cloned into this modified pCMV vector at the EcoRI and KpnI sites.

Full-length TPM1 plus 3′-UTR was amplified from MCF-7 cells using primers TPM1-EcoRI-5.1, 5′-GAATTCTGGACGCCATCAAGAAGAAGA-3′, and TPM1-UTR-NotI-3.1, 5′-GCGGCCGCCCTACAATGTGCATTTTATTCC-3′, then cloned into pCR8, and finally subcloned into the original pCMV-Myc. The 250-bp 3′-UTR region of TPM1 was also amplified from MCF-7 cells using primers TPM1-UTR-XbaI-5.1, 5′-TCTAGACTCTCAACGATATGACTTCCA-3′, and TPM1-UTR-XbaI-3.1, 5′-TCTAGATTTTTTTAGCTTACACAGTGTT-3′, using the same approach described above, and was finally cloned into pGL3 control vector (Promega, Madison, WI) at the XbaI site.

To construct a plasmid expressing the GFP-TPM1 fusion protein (pEGFP-TPM1+UTR), we also used primers TPM1-R1-5.1 and TPM1-UTR-NotI-3.1 as indicated above. This fragment was finally cloned into pEGFP-C3 (Clontech) at the EcoRI and NotI sites in-frame with the GFP coding region.

To clone the 3′-UTR of TPM1 into a GFP reporter, which was different from the GFP fusion construct, we first modified the pEGFP-C3 by introducing a stop codon in the front of the multiple cloning sites by PCR and then cloning the TPM1-UTR fragment into EcoRI site of this modified vector. All PCR products were verified by DNA sequencing before cloning into the final destination vectors.

Luciferase Assay—293T cells were seeded in 6-well plates and transfected with luciferase reporters using the calcium phosphate method as described above. After transfection, the cells were split into 12-well plates (in duplicates) and harvested for luciferase assays 24 h later using a luciferase assay kit (Promega) according to the manufacturer's protocol. β-Galactosidase was used for normalization.

Cell Growth Assay—After transfection with vector control or TPM1-expressing vector, the cells were seeded into 96-well plates at 2500 cell/well. The MTT assay was used to determine relative cell growth as described previously (21).

Anchorage-independent Assay—To determine anchorage-independent growth of transfected cells, the cells were grown in soft agar according to a published method (22). Briefly, 1 day after transfection with TPM1, cells were harvested and mixed with tissue culture medium containing 0.7% agar to result in a final agar concentration of 0.35%. Then, 1-ml samples of this cell suspension were immediately plated in 12-well plates covered with 0.6% agar in tissue culture medium and cultured at 37 °C with 5% CO_2. To assess cell viability before plating in soft agar, cell number was determined by trypan blue staining in Vi-Cell XR (Beckman Coulter, Fullerton, CA).

Western Blot—Total protein was isolated from tumor samples or 293T cells transfected with an appropriate plasmid in cell lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Protein concentration was measured using the Bio-Rad protein assay kit. The membrane was first probed with antibodies against Myc (Applied Biomaterials) or GFP

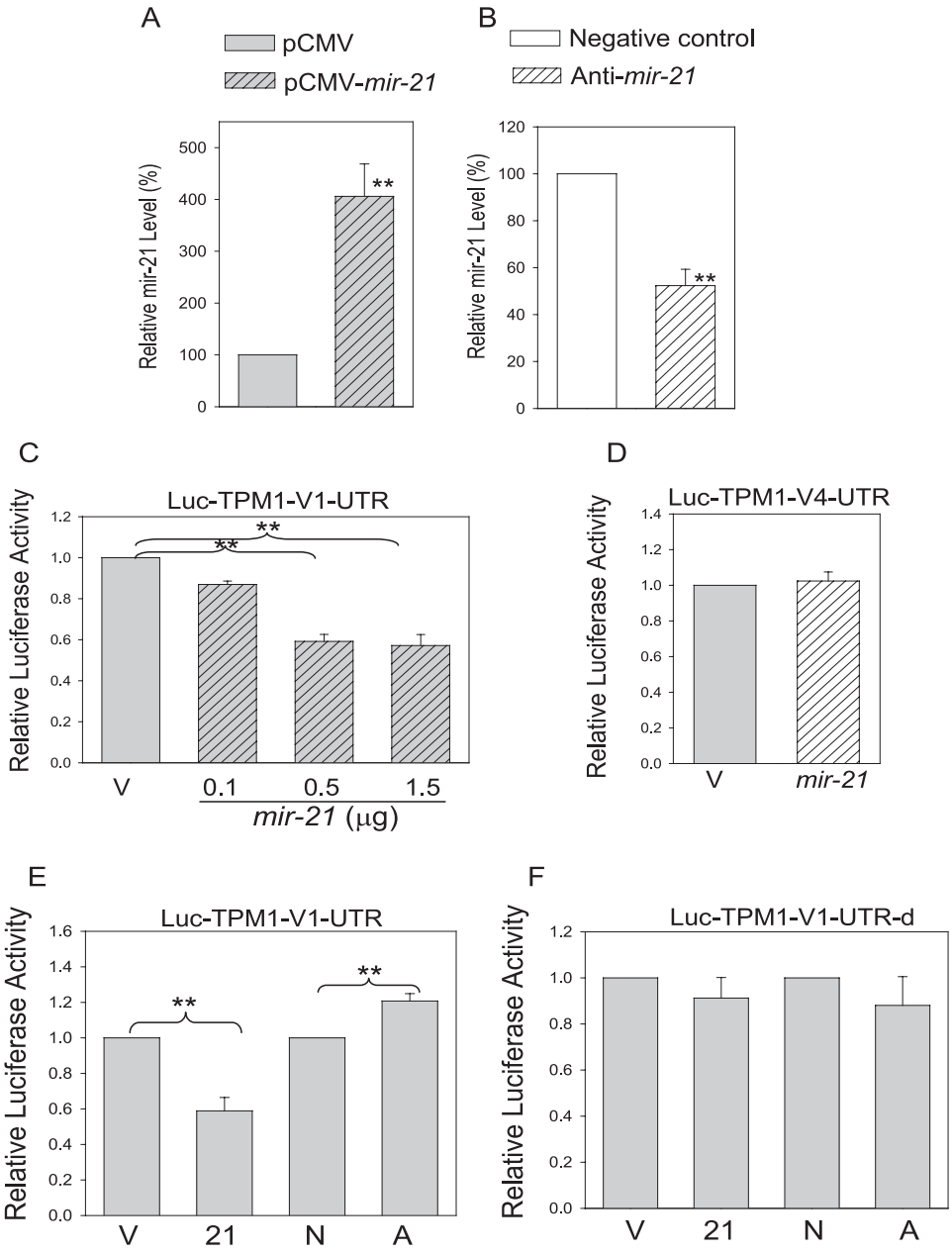

FIGURE 3. **The putative *mir-21* binding is responsible for *mir-21*-mediated down-regulation of luciferase activity.** *A* and *B*, detection of expression of mature *mir-21* in 293T cells 2 days after transfection with *mir-21*-expressing vector (1.0 μg) in a 6-well plate (*A*) or anti-*mir-21* (50 nM) (*B*) by TaqMan real-time PCR. *C*, suppression of Luc-TPM1-V1-UTR by *mir-21* in a dose-dependent manner. *D*, no effect is seen on the luciferase activity of Luc-TPM1-V4-UTR derived from variant 4 carrying no *mir-21* binding site. *E*, although *mir-21* suppresses, anti-*mir-21* increases the luciferase activity of Luc-TPM1-V1-UTR. *F*, deletion of the *mir-21* binding site abolishes the effect of *mir-21* on the luciferase activity. *V*, vector (pCMV); *21*, pCMV-*mir-21*; *N*, negative control oligonucleotide; *A*, anti-*mir-21*. **, $p < 0.01$.

(Clontech), and then with anti-β-actin antibody (Sigma-Aldrich). Secondary antibodies were labeled with either Alexa Fluor 680 (Invitrogen) or IRDye800 (Rockland Immunochemicals, Gilbertsville, PA). Signals were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Animal Work—Female nude (*nu*/*nu*) mice (4–5 weeks old) were purchased from Harlan (Indianapolis, IN) and were maintained in the Southern Illinois University School of Medicine's accredited animal facility. All animal studies were conducted in accordance with National Institutes of Health animal use guidelines and a protocol approved by the Southern Illinois University Animal Care Committee. Exponentially growing MCF-7 cells were harvested, mixed with 50% Matrigel (BD Biosciences) at 15 million cells/ml, and injected (1.5 million cells/spot) into mammary pads of female nude mice. To facilitate tumor growth, a 0.72-mg 17β-estradiol pellet (Innovative Research of America, Sarasota, FL) was implanted beneath the back skin. Tumors usually appeared 1 week after inoculation when anti-*mir-21* or negative control oligonucleotide was delivered to tumor sites by injecting 50 μl (50 nM) of the oligonucleotide carrying 12 μl of Optifect. One week later another injection of the same amount was performed. Tumor size was monitored every other day; 4 weeks after inoculation of MCF-7 cells, tumors were harvested, weighed, and frozen immediately in a −80 °C freezer.

Proteomic Analysis of Tumor Samples—Tumor samples that were harvested and stored at −80 °C were sent directly for 2-DIGE and mass spectrometry analysis, a service provided by Applied Biomics (Hayward, CA). Total protein was extracted and labeled with either Cy3 or Cy5. Isoelectric focusing in the first dimension was carried out at pH 3–10, and in the second dimension was carried out in 8–14% gradient SDS-PAGE. Differentially expressed proteins were cut out and subjected to trypsin digestion before mass spectrometry analysis.

Statistical Analysis—Data are expressed as means ±S.E., and $p < 0.01$ is considered as statistically significant by Student's *t* test.

RESULTS

Suppression of Tumor Growth by Anti-mir-21—We have previously shown that transient transfection of MCF-7 cells with anti-*mir-21* causes tumor growth inhibition in a xenograft carcinoma mouse model (21). Thus, we asked here whether intratumoral delivery of anti-*mir-21* has the same effect on tumor growth. Tumors treated with anti-*mir-21* grew substantially smaller in size than those treated with the negative control; tumors treated with anti-*mir-21* revealed a lower level of Ki-67 staining compared with the vector control (not shown). This is

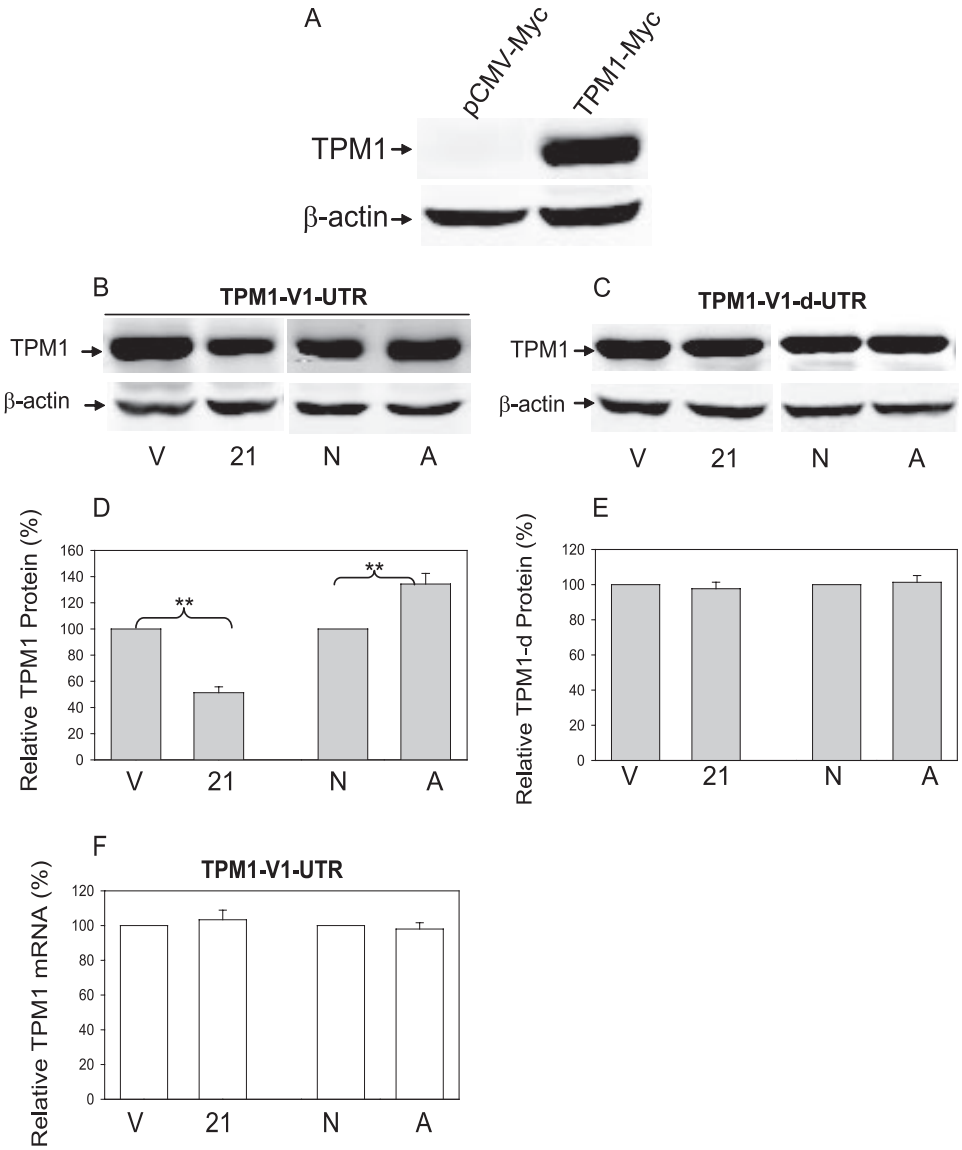

FIGURE 4. **Regulation of TPM1 expression by *mir-21* at the translational level.** *A*, expression of TPM1-Myc in 293T cells as determined by Western blot. The same membrane was first probed with anti Myc-antibody and then with anti β-actin as described under "Experimental Procedures." The effect of *mir-21* or anti-*mir-21* on the protein levels of TPM1-Myc (*B* and *D*) or TPM1-d (*C* and *E*), in which the *mir-21* binding site was deleted, is shown. *B* and *C* are representative of at least three separate experiments; *D* and *E* are the means of three separate experiments ± S.E. *F*, *mir-21* or anti-*mir-21* has no effect on the mRNA levels of TPM1-Myc, as determined by real-time qRT-PCR. *V*, vector (pCMV); *21*, pCMV-*mir-21*; *N*, negative control oligonucleotide; *A*, anti-*mir-21*. **, $p < 0.01$.

consistent with the previous finding (21), suggesting that suppression of tumor growth likely occurs because of reduced cell proliferation, increased apoptosis, or both as suggested previously (21, 23). Therefore, these results not only support the notion that *mir-21* is an oncogenic miRNA but also imply that anti-*mir-21* has a therapeutic potential.

TPM1 Is Up-regulated in Anti-mir-21-treated Tumor Samples as Detected by 2-DIGE Analysis—Although *mir-21* is overexpressed in many types of tumors, suggesting its role in cancer development, the underlying mechanism of *mir-21*-mediated tumorigenesis is still unclear largely because of limited knowledge about *mir-21* targets. Although various computer-aided algorithms have predicted many putative *mir-21* targets, these targets have not been validated experimentally. Because miRNAs are believed to regulate gene expression mainly through translational repression in mammalian cells, we thought to determine the differential expression of proteins from the tumor samples after treatment with anti-*mir-21*. Protein was extracted from tumors derived from MCF-7 cells treated with either the negative control (labeled with Cy3) or anti-*mir-21* (labeled with Cy5). Unlike conventional two-dimensional gels in which two samples are run in separate gels, this method separates two samples labeled with different fluorescent dyes in a single gel, thus eliminating gel-to-gel variation and allowing for easy comparison of relative expression levels. After separating the proteins by isoelectric focusing and SDS-PAGE, we found that several proteins were either up-regulated or down-regulated as shown by either *red* or *green* color, respectively (Fig. 1). This result, in fact, is in agreement with the finding that *mir-122* also causes up-regulation or down-regulation of many proteins (24), because some of these differentially expressed proteins may be due to the secondary effect of miRNA regulation. Analysis of another pair of tumor samples harvested from different mice revealed an almost identical pattern to that of Fig. 1, suggesting the reproducibility of this method. We are particularly interested in those proteins up-regulated by anti-*mir-21* because they are potential direct targets for *mir-21*. We picked 10 protein spots that were up-regulated more than 2-fold in the tumor samples treated with anti-*mir-21* compared with the negative control; these are *circled* in Fig. 1*B*. Mass spectrometry analysis identified seven of them with a good score. Among them, three proteins have been implicated in tumorigenesis: TPM1 (25), integrin-β4-binding protein (ITGB4BP), (26) and selenium-binding protein-1 (SELENBP1) (27). Therefore, we tested these three genes by cloning their UTRs into a luciferase reporter. Interestingly, Western blot analysis of the tumor samples also indicated that the endogenous TPM1 was increased in the anti-*mir-21*-treated tumors by almost 2-fold (Fig. 1, *C* and *D*). Further characterization identified TPM1 as a direct target for *mir-21* as described below.

TPM1 Carries a Putative mir-21 Binding Site, Which Is Responsible for Regulation by mir-21—The tropomyosins (TMs or TPMs) are a group of proteins that bind to the sides of actin filaments; there are at least four separate proteins, TPM1, -2, -3,

and -4, encoded by different genes (28). TPM1 has seven variants through alternative splicing. Coincidentally, TPM1 variants 1 and 5 carry a putative *mir-21* binding site, as predicted by the Sanger miRNA data base target search program (Fig. 2*A*). Variant 1 differs from variant 5 in a sequence coding for 24 amino acids and also by lacking an additional 48 nucleotides upstream of the 3′-UTR (Fig. 2*A*). The potential base pairing between *mir-21* and TPM1 3′-UTR is shown in Fig. 2*B*. Thus, we tried to amplify this UTR region of both variants from MCF-7 cells, which, however, appeared to express only TPM1 variant 1. Hence, we cloned this variant 1 3′-UTR into pGL3 control vector. As shown in Fig. 2*C*, the luciferase activity in 293T cells for Luc-TPM1-V1-UTR was about 20% less than that of pGL3 control vector, suggesting that TPM1 3′-UTR carries a regulatory element(s).

To confirm that this regulatory region is *mir-21* specific, we transfected 293T cells with the same Luc-TPM1-UTR plasmid along with either the pCMV vector or the *mir-21*-expressing plasmid. The ectopic expression of *mir-21* was confirmed by TaqMan real-time PCR, which revealed about a 4-fold higher *mir-21* expression in the *mir-21*-transfected cells than in vector control (Fig. 3*A*). In contrast, anti-*mir-21* reduced *mir-21* by almost 50%, as determined by the same method (Fig. 3*B*). We then transfected the 293T cells with various amounts of *mir-21*-expressing vector. As shown in Fig. 3*C*, reduction of luciferase activity by *mir-21* was dose-dependent, suggesting that this regulation is specifically responsive to *mir-21*. In contrast, *mir-21* had no effect on Luc-TPM1-V4-UTR, which is derived from variant 4 and lacks the *mir-21* binding site (Fig. 3*D*). In addition, we tested the effect of anti-*mir-21* on the luciferase activity of Luc-TPM1-V1-UTR. As expected, *mir-21* suppressed the luciferase activity, whereas anti-*mir-21* increased the luciferase activity (Fig. 4*E*), further suggesting that expression of TPM1 is specifically regulated by *mir-21*. To determine the role of the *mir-21* binding site in regulating its expression, we deleted the *mir-21* binding site in variant 1 (Luc-TPM1-V1-UTR-d). As shown in Fig. 3*F*, neither *mir-21* nor anti-*mir-21* had any effect on the luciferase activity, highlighting the importance of this *mir-21* binding site.

To ensure that down-regulation of luciferase activity by *mir-21* was not due to the reporter we used, we made similar reporter constructs in EGFP vector. In this case, we cloned TPM1-V1-UTR into the EcoRI site of the modified pEGFP-C3 (see "Experimental Procedures"). Consistent with the luciferase data, the level of EGFP-TPM1-V1-UTR was reduced by *mir-21* but was increased by anti-*mir-21*, as measured either by Western blot or fluorescence microscopy (not shown).

mir-21 Regulates TPM1 at the Translational Level—Translational repression is a major mechanism of miRNAs to regulate gene expression (29). To determine whether *mir-21* also suppresses TPM1 through translational repression, we cloned the full-length TPM1 plus the 3′-UTR into pCMV-Myc. Expression of Myc-tagged TPM1 was confirmed by anti-Myc antibody (Fig. 4*A*). Importantly, although ectopic expression of *mir-21* significantly reduced TPM1 protein, anti-*mir-21* enhanced TPM1 protein (Fig. 4, *B* and *D*). To further determine the importance of the *mir-21* binding site, we did similar experiments with pCMV-Myc-TPM1+UTR-d in which the *mir-21*-binding site was deleted. Deletion of this site abolished the effect of *mir-21* or anti-*mir-21* on TPM1 expression at the protein level (Fig. 4, *C* and *E*). However, despite the effect of *mir-21* or anti-*mir-21* on TPM1 at the protein level, no effect on the TPM1 mRNA level was detected by real-time qRT-PCR for pCMV-Myc-TPM1+UTR (Fig. 4*F*). Therefore, these results suggest that the *mir-21* binding site present in the TPM1-UTR region is critical for *mir-21*-mediated regulation at the translational level.

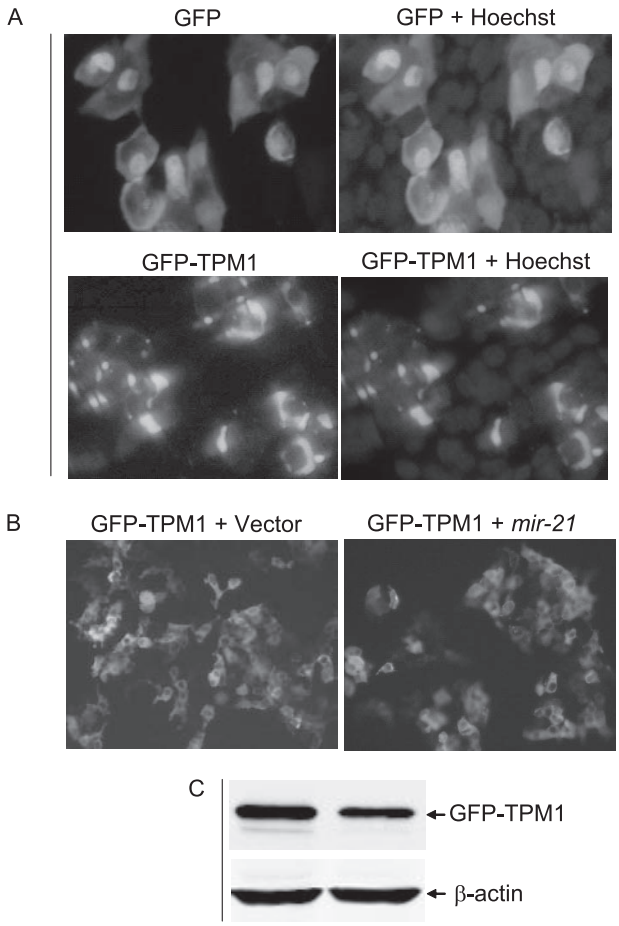

FIGURE 5. **Regulation of expression of the GFP-TPM1 fusion protein by *mir-21*.** *A*, expression of GFP or GFP-TPM1 in 293T cells. The plasmids pEGFP-C3 and pEGFP-TPM1+UTR were first introduced into 293T cells. One day later, cells were seeded on coverslips and grown for 16 h. Cells were stained with Hoechst dye and examined under a fluorescence microscope. Note that GFP-TPM1 fusion protein is present exclusively in the cytoplasm as compared with GPF, which is present throughout the cell. *B* and *C*, effect of *mir-21* on expression of the GFP-TPM1 fusion protein as determined by fluorescence microcopy (*B*) or Western blot (*C*). Co-transfection with β-galactosidase-expressing vector indicated a comparable transfection efficiency between GFP-TPM1+UTR with vector control and GFP-TPM1+UTR with *mir-21*.

In addition, we made a GFP fusion construct with the full-length TPM1 plus the 3′-UTR (GPF-TPM1+UTR). We first confirmed its expression of its fusion protein by Western blot (not shown). Fluorescence microscopy clearly showed a distinguished subcellular localization of GFP-TPM1. Although we detected the green protein all over the cell for GFP alone, GFP-TPM1 fusion protein was localized exclusively to the cytoplasm (Fig. 6*A*). Moreover, *mir-21* also reduced the GFP fusion protein expression, as detected by fluorescence microscopy (Fig.

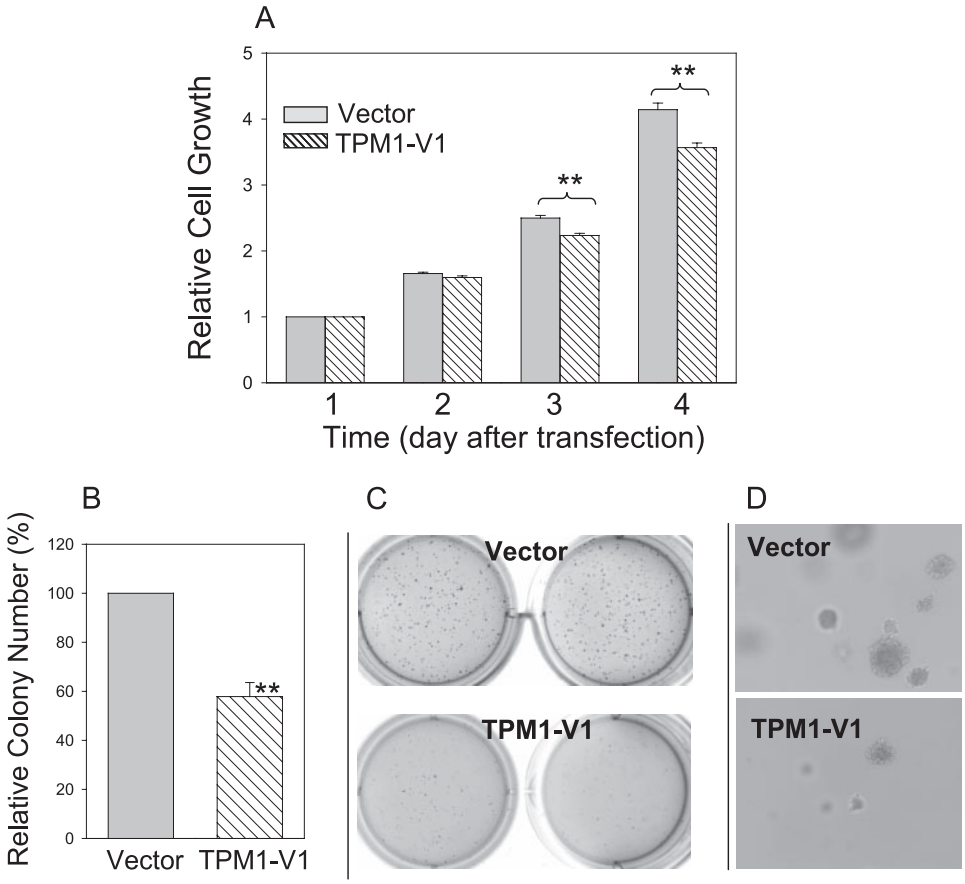

FIGURE 6. **Overexpression of TPM1-V1 suppresses cell growth and colony formation on soft agar.** *A, in vitro* cell growth curve. MCF-7 cells were transfected with either pCMV-Myc vector control or pCMV-Myc-TPM1-V1. Relative cell growth was determined at the indicated times by MTT assay as described under "Experimental Procedures." *B–D*, soft agar assay. *A* and *B*, values are means ± S.E. of three separate experiments. **, $p < 0.01$. *C* and *D*, representative pictures of colonies at low magnification (*C*) and high magnification (*D*). Note that colonies are smaller for pCMV-Myc-TPM1-V1 than for vector control.

5*B*), which was confirmed by Western blot (Fig. 5*C*). In this case, we co-transfected the cells with GFP-TPM1 and β-galactosidase plasmid, confirming a comparable transfection efficiency between GFP-TPM1+UTR with vector and GFP-TPM1+UTR with *mir-21*. Thus, even though the *mir-21* binding site is away from the GFP (separated by TPM1 coding region), it is still functional. This result further indicates that TPM1 is a *mir-21* target.

Overexpression of TPM1 Suppresses Cell Growth in Vitro and Anchorage-dependent Growth—Because previous studies have indicated that suppression of TPM1 is a prominent feature of many transformed cells, and TPM1 functions as a tumor suppressor (30), we first tested whether overexpression of TPM1-V1 affects cell growth. Thus, pCMV-Myc-TPM1-V1 was transiently transfected to MCF-7 cells and their growth determined by MTT assays. We found that overexpression of TPM1-V1 suppressed cell growth in a time-dependent manner (Fig. 6*A*). For instance, although there was no difference between vector control and TPM1-V1 during the first 2 days after transfection, by days 3 and 4 after transfection we detected that cells transfected with TPM1-V1 grew more slowly than the vector control, with about 20% inhibition. To determine whether TPM1-V1 affects anchorage-independent growth, we grew MCF-7 cells transfected with either vector control or pCMV-Myc-TPM1-V1 in the soft agar medium. As shown in Fig. 6*B*, the number of colonies from MCF-7 cells transfected with pCMV-Myc-TPM1-V1 was significantly lower than that of vector control. Of interest, although *in vitro* cell growth inhibition was about 20%, a greater effect was seen on inhibition of colony formation (almost 50%; Fig. 6*B*). Furthermore, the size of the colonies from the cells transfected with pCMV-Myc-TPM1-V1 was much smaller than those of vector control (Fig. 6, *C* and *D*). These results are consistent with the finding that expression of TPM1 induces anoikis (30), thus providing further evidence that TPM1 is a tumor suppressor. Accordingly, identification of TPM1 as *mir-21* target gene may explain at least in part why suppression of *mir-21* can inhibit tumor growth, as we have demonstrated previously (21).

DISCUSSION

It is now well known that miRNAs regulate a variety of cellular pathways through regulation of expression of multiple target genes (4). In this regard, *mir-21* has been suggested to function as an oncogene because it is overexpressed in many types of tumors compared with the normal tissues (21, 23, 31, 32). Furthermore, suppression of *mir-21* inhibits cell growth, possibly through activation of apoptosis pathways (21, 23). However, it largely remains to be determined as to how a specific miRNA affects these pathways, in particular, regarding miRNA-associated oncogenesis, because little is known about the physiologic targets of *mir-21*. Our study indicates that TPM1 is one such target. As a tumor suppressor, TPM1 has been shown to play a role in suppression of the malignant phenotype (25, 33, 34). Thus, identification of TPM1 as a *mir-21* target gene provides a possible explanation of why suppression of *mir-21* can inhibit tumor growth (21).

In animals, miRNAs are believed to bind through partial homologous sequence to a target gene at 3′-UTR, causing translational repression. This notion is supported by two well characterized miRNA target genes that play a critical role in cancer, *ras* and *bcl-2*. In the former case, *let-7* binds to the 3′-UTR of *ras* and causes its translational repression by 8 bases of homology (11). Similarly, *mir-16* directly targets *bcl-2* at the 3′-UTR by a same mechanism (35). Apparently, both are tumor-suppressive miRNAs. With regard to oncogenic miRNAs, a relatively limited number of target genes

has been characterized experimentally, although there is overwhelming information on putative targets predicted by different algorithm programs. For instance, the Sanger miRNA data base target search reveals >900 targets for *mir-21*, which is not consistent with the prediction of about 100 target genes per single miRNA (36). Furthermore, we previously tested several of the putative *mir-21* targets such as FasL and CDC25A by Western blot, but none of them seem to be regulated by *mir-21* (21). Therefore, it is very likely that only a small fraction of predicted targets may be true targets, and thus it would be a daunting task to validate them. Accordingly, we took an alternative approach, *i.e.* proteomics, because a major action of miRNAs is thought to be at the translation (29).

Several lines of evidence indicate that TPM1 is a *mir-21* target. First, TPM1 expression is increased in tumors treated with anti-*mir-21*. Second, the ability of *mir-21* to regulate TPM1 protein expression is likely direct, as it binds to the 3′-UTR region of TPM1 mRNA with complementarity to the *mir-21* seed region (Fig. 2*B*). Third, Luc-TPM1-V1-UTR is specifically responsive to *mir-21* overexpression or anti-*mir-21*. Finally, deletion of the *mir-21* site abolishes its *mir-21* regulation. Although miRNAs may regulate protein expression by accelerating messenger RNA degradation and/or inhibiting transcription from existing messenger RNA (37), our results suggest that *mir-21* inhibits TPM1 protein translation, as steady state TPM1 mRNA levels are not affected by *mir-21* or anti-*mir-21*.

Tropomyosins are widely distributed in all cell types associated with actin such that they serve as actin-binding proteins and stabilize microfilaments (38). In animals, four known tropomyosin genes code for diverse isoforms that are expressed in a tissue-specific manner and regulated by an alternative splicing mechanism (28). Suppression of TPM1 and TPM2 has been reported in malignant cells, suggesting a role for these proteins in neoplastic transformation (25, 39). In addition, transfection of tropomyosins into viral oncogene-transformed rodent cells suppresses tumorigenic phenotypes (40, 41). Moreover, TPMs regulate both microfilament organization and anchorage-independent growth, highlighting the importance of TPMs in cell transformation (33). TPMs belong to the class II tumor suppressor genes (42), because expression of TPMs is apparently subject to epigenetic regulation (43, 44); these genes are structurally intact in their sequences but are underexpressed or unexpressed due to down-regulation or silencing in transcription or translation.

Epigenetic modification of TPM expression seems to involve several cellular factors. One such factor is methylation. For instance, treatment of cancer cells with demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) increases mRNA levels of TPM1 (43). Importantly, such treatment can restore transforming growth factor-β induction of TPM1 and formation of stress fibers, thus altering the transforming growth factor-β tumor suppressor function (43). Interestingly, MCF-7 cells express little TPM1 (34), and inhibition of DNA methyl transferase with 5-aza-dC alone does not induce TPM1 expression (44). However, combined treatment of the histone deactylase inhibitor trichostatin A and 5-aza-dC results in readily detectable expression of TPM1 (44), suggesting that acetylation may also be involved in regulating TPM1 expression. Thus, this study provides another potential mechanism of posttranscriptional regulation of TPM1 expression, ultimately modulating cell transformation and tumor cell growth.

Interestingly, the 3′-UTR region of TPMs alone may also play a role in tumor suppression. For instance, constitutive expression of RNA from the 3′-UTR suppresses anchorage-independent growth and tumor formation in a nondifferentiating mutant myogenic cell line (45), although the 3′-UTR of TPMs alone may not be sufficient to cause tumor suppression or may not be required for tumor suppression in other types of cells (46, 47). Nevertheless, it would be of interest to determine whether oncogenic miRNAs such as *mir-21* interact with this region. If this interaction exists, we would expect that overexpression of this 3′-UTR region might deplete a pool of such miRNAs in the cell, leading to tumor suppression.

In summary, TPM1 expression can be regulated by *mir-21*. This study extends our knowledge about the regulation of TPM1, a tumor suppressor protein. Thus, in addition to epigenetic regulation, as mentioned above, TPM1 is also regulated at the translational level by miRNAs. Given that a single miRNA has multiple targets, we believe that *mir-21* also has many targets. It is our expectation that more *mir-21* targets will be identified in the near future with the same proteomic approach such that we will be better able to understand the molecular basis of *mir-21*-mediated tumorigenesis.

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